Structure and Function of the N-Cadherin/Catenin Complex in Retinoblastoma

Elisabeth H. Van Aken,[1] Peggy Papeleu,[2] Patrick De Potter,[3] Erik Bruyneel,[2] Jan Philippé,[4] Stefan Seregard,[5] Anders Kvanta,[5] Jean-Jacques De Laey,[1] and Marc M. Mareel[2]

Purpose. To identify in human retinoblastoma and normal retinal tissue the type of cadherin, its relationship with cytoplasmic catenins, and its participation in invasion.

Methods. The cadherin/catenin complex was characterized in surgical retinoblastoma specimens from five patients and human retinas from four donor eyes by immunocytochemistry, flow cytometry, and coimmunoprecipitation with antibodies against N-cadherin, α-catenin, and β-catenin, followed by Western blot analysis or autoradiography. Y79 and WERI-Rb-1 retinoblastoma cell lines serve the evaluation of the cadherin/catenin complex in aggregation and collagen type I invasion in vitro. The association of the cadherin/catenin complex with the cytoskeleton was examined by an antibody-capping assay.

Results. In retinoblastoma and normal retina N-cadherin associated with α-catenin and β-catenin but not E- or P-cadherin. The N-cadherin/catenin complex formed a regular, linear, and continuous honeycomb pattern in normal retina that was irregular, clustered, and interrupted in retinoblastoma. The N-cadherin/catenin complex was found also in the retinoblastoma cell lines WERI-Rb and Y79, in which it also showed an irregular pattern. Both cell lines were invasive in collagen type I, and invasion was inhibited by the GC-4 antibody, which functionally neutralizes N-cadherin. Less GC-4 antibody was needed to inhibit invasion of Y79 cells, which expressed N-cadherin at a lower level, than to inhibit invasion of WERI-Rb-1 cells. In both cell lines, antibody capping of the N-cadherin/catenin complex indicated that its linkage with the cytoskeleton were weak or absent.

Conclusions. Retinoblastoma cells, in contrast with normal retina, express an N-cadherin/catenin complex that is irregularly distributed and weakly linked to the cytoskeleton. In retinoblastoma, this complex acts as an invasion promoter. (*Invest Ophthalmol Vis Sci.* 2002;43:595–602)

Retinoblastoma originates from the inner nuclear layer of the retina. It is the most common intraocular tumor in children, occurring with a frequency of 1 in 20,000 births.[1,2] The development of retinoblastoma is initiated by deletion or mutational inactivation of the *RB* tumor-suppressor gene on chromosome 13q14, encoding a protein that interacts with E2F transcription factors and regulates transcription of genes required for progression through the cell cycle.[3] RB-deficient neuroblasts lose control of proliferation, but still differentiate and form a retinoma. Additional mutations—for example, in *Notch* family genes— cause loss of differentiation, resulting in retinoblastoma.[2] The degree of invasion of retinoblastomas into ocular coats and optic nerve is the main prognostic factor.[4] Transgenic mice with one *RB* allele inactivated do not form retinoblastomas, whereas the double-knockout $RB^{-/-}$ mice are not viable.[5,6] Transgenic mice, carrying the simian virus (SV)40-T oncogene under the control of promoters for eye-specific genes encoding interphotoreceptor retinoid-binding protein or opsin, show development of tumors resembling human retinoblastomas.[7] Similar lesions were obtained after injection of human retinoblastoma cell lines Y79 and WERI-Rb-1 into the subretinal space of adult Fischer 344 rats[8] or into the anterior chamber of nude mice.[9] Injection of Y79 into the vitreum of mice resulted in direct invasive spread to the optic nerve, the brain, and contralateral optic nerve, whereas WERI-Rb-1 cells invaded the anterior structures of the eye.[4]

Cadherins compose a superfamily of transmembrane molecules that require calcium for their structure and function. Members of the classic type I cadherin subfamily have a conserved histidine-alanine-valine (HAV) cell adhesion recognition sequence in their first extracellular domain[10]; Examples are the N(euronal)- and E(pithelial)-cadherins.[11] The catenins link the cytoplasmic domain of classic cadherins to the cytoskeleton. E-cadherin is an invasion suppressor, as demonstrated in experimental and human cancer.[12–14] N-cadherin was originally found in neuronal tissues,[15] and later also in lens, skeletal, and heart muscle cells[16]; osteoblasts[17]; and fibroblasts,[18] where it serves as an essential cell–cell adhesion molecule. Transient N-cadherin expression is associated with rearrangement and invasion of cells during morphogenesis.[19] In prostate,[20] breast,[21] and head and neck squamous cell carcinomas,[22] and in melanomas,[23] N-cadherin expression and increased invasiveness are associated with loss of a functional E-cadherin/catenin complex. The latter has been explained through increased motility of cancer cells when attached to N-cadherin–positive stromal cells.[22,24] The role of N-cadherin in cells migrating from N-cadherin–positive normal tissues is less well understood. When migrating, neural crest cells accumulate the bulk of N-cadherin molecules in the detergent-soluble fraction, and when restoring intercellular contacts, N-cadherin molecules are recruited to the adherens junctions.[25,26] The developing retinas in chick and mice served as models for neurite outgrowth. In the models just described, direct heterophilic interaction between an HAV-binding motif on the fourth extracellular domain of N-cadherin and an HAV motif on fibroblast growth factor receptor (FGFR)-1[27] and also interaction between N-cadherin and β1 integrin complexes through phosphorylation cascades[28,29] mediate neurite outgrowth.

So far, N-cadherin has not been investigated in normal human retina or in retinoblastoma. In Y79 and WERI-Rb-1

From the Departments of [1]Ophthalmology and [4]Clinical Chemistry, Microbiology, and Immunology and the [2]Laboratory of Experimental Cancerology, Ghent University Hospital, Gent, Belgium; the [3]Ocular Oncology Unit, Cliniques Universitaires St-Luc, Brussels, Belgium; and the [5]Ophthalmic Pathology and Oncology Service, St. Erik's Eye Hospital, Stockholm, Sweden.

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Corresponding author: Marc M. Mareel, Laboratory of Experimental Cancerology, Ghent University Hospital, De Pintelaan 185, B-9000 Gent, Belgium; marc.mareel@rug.ac.be.

retinoblastoma cell lines a positive correlation was found between calcium-dependent aggregation and the amount of N-cadherin expressed.[30] In developing mouse and avian retinas, N-cadherin is initially expressed in all undifferentiated retinal cells. Later, steady loss of expression results in exclusive localization of N-cadherin at the outer limiting membrane in the chick[19,31] or at the inner nuclear layer in the mouse.[32] It was our goal to study N-cadherin in human retinoblastoma by immunohistochemistry and immunoprecipitation. The retinoblastoma cell lines Y79 and WERI-Rb-1 were used to analyze the putative invasion–stimulatory function of the N-cadherin/catenin complex.

Materials and Methods

Tissues and Cell Lines

Five samples of retinoblastoma (volume between 10 and 40 mm^3) were obtained from the operating theater and immediately snap frozen in liquid nitrogen, with or without isopentane. Normal retina was dissected from four donor eyes, obtained within 24 hours of death, that were to be used for corneal transplantation. Choroid and retinal pigment epithelium were dissected and snap frozen together with the retina for correct orientation and sectioning. The human retinoblastoma cell lines Y79[33] and WERI-Rb-1[1] (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Gibco, Merelbeke, Belgium), supplemented with 15% fetal calf serum (FCS; Gibco), 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Human neuroblastoma cells SK-N-SH[34] served as a positive control in molecular, immunocytochemical, and functional analyses of human N-cadherin–expressing cells.[35] ARM cells, which are mouse sarcoma cells transfected with chicken cDNA encoding N-cadherin, were used as a positive control for invasive cells expressing N-cadherin.[36] The dog kidney epithelial cells MDCK/AZ[37] and the spontaneously immortalized rat enteric myofibroblasts DHD-Fib[38] were used as noninvasive and invasive controls, respectively, in the collagen type I invasion assay.

Antibodies and Reagents

Rabbit polyclonal antibodies (Sigma, Bornem, Belgium), raised against a peptide homologous to the C-terminal part (amino acids 890-901) of human α-catenin (the same for αE and αN) and against a peptide corresponding to the C-terminal part (amino acids 768-781) of human β-catenin, were used for immunoprecipitation, immunostaining on Western blot (diluted 1:1000), and immunocytochemistry (diluted 1:500). The mouse monoclonal antibody recognizing the extracellular domain of both chicken and human N-cadherin (A-CAM, clone GC-4; Sigma, St. Louis, MO) was used for immunocytochemistry (diluted 1:30). A purified mouse monoclonal IgG1 clone (11711.11; R&D Systems, Minneapolis, MN) was used as isotype control antibody in the collagen type I invasion assay. GC-4 without azide[39] was used for the capping assay (diluted 1:30) and the collagen type I invasion assay (at different dilutions, as described in the Results section). The mouse monoclonal antibody raised against the human N-cadherin cytoplasmic domain (13A9), kindly provided by Margaret J. Wheelock (Department of Biology, University of Toledo, Toledo, OH), was used for immunoprecipitation and immunostaining on Western blot (diluted 1:500). The rabbit polyclonal antibody CH-19 (diluted 1:1000; Sigma), raised against the C-terminal part of chicken N-cadherin; the monoclonal antibody HECD-1 (diluted 1:1000), recognizing the extracellular domain of E-cadherin (Takara, Kyoto, Japan); and the monoclonal antibody raised against a peptide homologous to the N-terminal part (amino acids 72-259) of P-cadherin (diluted 1:1000; BD Bioscience, HQ Pharmingen, Schwechat, Austria), were used for immunostaining on Western blot.

Immunoprecipitation, Gel Electrophoresis, Western Blot analysis

For immunoprecipitation, lysates were prepared from tumor fragments and cell cultures in phosphate-buffered saline (PBS) containing 1% Triton X-100, 1% NP-40, and the following protease inhibitors (all from Sigma): 1.72 mM phenylmethylsulfonyl fluoride, 21 μM leupeptin, and 10 μg/mL aprotinin. Equal amounts of protein were incubated with protein G Sepharose beads for 1 hour, the beads were discarded, and the supernatant was incubated with primary antibody (1 μg/precipitation) for 3 hours at 4°C followed by protein G Sepharose beads for 1 hour. Precipitated proteins were dissolved in sample buffer,[40] boiled in the presence of 5% mercaptoethanol, separated by 7.5% SDS-PAGE, and transferred onto membranes (Immobilon-P; Millipore Corp., Bedford, MA). After quenching with 5% nonfat dry milk containing 0.5% Tween-20, the membranes were incubated with primary antibody, followed by three washes for 5 minutes each and incubation with either alkaline phosphatase–conjugated secondary antibodies (Sigma) using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate or horseradish peroxidase–conjugated secondary antibodies with enhanced chemiluminescence (ECL) reagent (Amersham Life Science, Buckinghamshire, UK) as a substrate. For metabolic labeling, cells were incubated for 4 hours with 100 μCi/mL Tran^{35}S label, containing 70% L-methionine, [^{35}S] (ICN Biomedicals, Costa Mesa, CA) in methionine- and cysteine-free medium containing 2% FCS, followed by washing and extraction in PBS containing 1% Triton X-100, 1% NP-40, 1.72 mM phenylmethylsulfonyl fluoride, 21 μM leupeptin, 10 μg/mL aprotinin, 0.9 mM $CaCl_2$, and 0.334 mM $MgCl_2$. After lysates were diluted to contain equal amounts of trichloroacetic acid–precipitable radioactivity, immunoprecipitation, gel electrophoresis, and Western blot analyses were further used as as for nonmetabolic labeled cells. Blots containing ^{35}S methionine–labeled proteins were exposed to autoradiographic film.

Immunocytochemistry and Immunohistochemistry

Subconfluent cultures of SK-N-SH cells on glass coverslips were fixed in methanol (−20°C) for immunocytochemical staining. Aspecific binding of the primary antibody was blocked by incubation for 30 minutes with 5% bovine serum albumin. Subsequently, SK-N-SH cells were incubated with the primary antibody at room temperature for 90 minutes, followed by FITC-labeled anti-mouse (diluted 1:20) or biotinylated anti-rabbit immunoglobulins (diluted 1:50; Amersham) for another 90 minutes. Thereafter, cells were incubated for 15 minutes with Texas red–conjugated streptavidin (diluted 1:50) and 4′,6-diamino-2-phenylindol (DAPI, 0.4 mg/mL; Sigma) to stain the nuclei. Coverslips were mounted in aqueous medium (Glycergel; Dako, A/S, Glostrup, Denmark), examined by fluorescence microscopy (Dialux 20; Leitz, Wetzlar, Germany), and photographed with a camera system (Orthomat E; Leitz). Y79 and WERI-Rb-1 cells were stained in suspension. Therefore, 1.2 million cells/mL were washed with PBS, followed by fixation with paraformaldehyde 3%. For staining of α- and β-catenin, Y79 and WERI-Rb-1 cells were permeabilized with Triton X-100 (0.2%). Cells were immunostained, as described for SK-N-SH cells. After immunostaining, 20 μL of Y79 and WERI-Rb-1 cell suspensions were either dried on poly-L-lysine–coated coverslips, mounted in gelatin, and photographed with the camera system (Orthomat E; Leitz) or analyzed by flow cytometry (FACSort; BD Biosciences; Mountain View, CA). Flow cytometric results were analyzed with Kolmogorov-Smirnov statistics.

For capping experiments on N-cadherin at the cell surface, SK-N-SH, WERI-Rb-1, and Y79 cells were treated in suspension. Therefore, SK-N-SH cells were first detached in cadherin-saving conditions.[41] Briefly, cells were detached by collagenase A (Roche Molecular Biochemicals, Mannheim, Germany) followed by trypsin, both in presence of Ca^{2+} at 0.04 mM. Unfixed SK-N-SH, WERI-Rb-1, and Y79 cell suspensions were then treated further at 4°C.[42] Cells were washed with PBS, treated for 1 hour with GC-4 antibody without azide, spun for 2 minutes at 350 g, and treated with FITC-labeled anti-mouse immuno-

globulins for 30 minutes. Cells were further incubated at 37°C, and the fluorescent signal was evaluated microscopically every 10 minutes.

For immunohistochemical staining, 5-μm-thick sections from isopentane-coated frozen retinoblastoma and nontumorous retina were fixed in methanol and stained as described for immunocytochemical staining of SK-N-SH cells seeded on coverslips. Immunostained sections were mounted in aqueous medium (Glycergel; Dako).

Functional Assays

The collagen type I invasion assay was performed as described.[43] Briefly, collagen G (type I solution; Seromed, Biochrom KG, Berlin, Germany) was dissolved at 0.22% in bicarbonate buffer, containing DMEM. Aliquots (1.2 mL) were poured into a six-well plate and incubated overnight at 37°C for gelation. Cells were seeded on top of the collagen gel, with or without GC-4 antibody, against N-cadherin. After a 24-hour incubation at 37°C, the number of cells that invaded the gel was counted under a phase-contrast microscope with a computer-controlled step motor.[44] The invasion index was expressed as the number of cells inside the gel relative to the total number of cells. Student's *t*-test (95%) was used for statistical analysis. A fast aggregation assay was performed as described previously.[41] Single-cell suspensions were prepared in accordance with a cadherin-saving procedure and incubated in an isotonic buffer containing 1.25 mM Ca^{2+} under shaking (Gyrotory; New Brunswick Scientific, New Brunswick, NJ) at 80 rpm for 30 minutes, with or without GC-4 antibody against N-cadherin (diluted 1:50). Particle diameters were measured in a particle size counter (LS 200; Coulter, Lake Placid, NY) at the start (N_0) and after 30 minutes of incubation (N_{30}) and plotted against percentage volume distribution.

Results

N-Cadherin/Catenin Complex in Human Retinoblastoma Compared with Normal Retina

Normal retina tissues (4 of 4 samples) expressed N-cadherin, α-catenin, and β-catenin. The expression pattern of N-cadherin was not limited to the outer limiting membrane, as in the chick,[19,31] or to the inner nuclear layer, as in the mouse,[32] but was found in the inner and outer nuclear layers, as well as the outer limiting membrane. α-Catenin and β-catenin were found in the same layers of the retina as N-cadherin, and all were organized in a regular honeycomb pattern, as illustrated in Figure 1. In retinoblastoma, the honeycomb pattern of N-cadherin, α-catenin, and β-catenin was irregular, with clusters of the three proteins throughout the tissue. In none of the investigated retinoblastomas was β-catenin seen in the nucleus. E- or P-cadherin was not expressed in normal retina or in retinoblastoma (data not shown). Immunostaining on Western blot confirmed the qualitative immunohistochemical findings. Moreover, N-cadherin, α-catenin, and β-catenin showed less immunoreactivity per unit protein weight in retinoblastoma than in normal retina. A pancadherin antibody, raised against the C-terminal part of chicken N-cadherin and recognizing class I cadherins, showed the same reactivity on Western blot as the 13A9 antibody that specifically recognized N-cadherin. It was concluded that N-cadherin, α-catenin, and β-catenin form a complex in both retinoblastoma and normal retina, in that not only was N-cadherin precipitated with β-catenin, but β-catenin was precipitated with N-cadherin, and α-catenin was associated with β-catenin and N-cadherin immunoprecipitates.

Characterization of the Cadherin/Catenin Complex in Y79 and WERI-Rb-1 Cells

To investigate the function of N-cadherin in cellular invasion, the Y79 and WERI-Rb-1 cell lines were used. N-cadherin, α-catenin, and β-catenin were found in all lysates from SK-N-SH and WERI-Rb-1 cells, which indicated that the three proteins are organized in an N-cadherin/catenin complex (Fig. 2A). The immunoreactivity of the N-cadherin/catenin complex in WERI-Rb-1 cells was less intense than in SK-N-SH cells. Because immunostaining of Western blot analysis of total cell lysates from WERI-Rb-1 cells also showed lesser reactivity than total cell lysates from SK-N-SH cells (data not shown), it was concluded that the fainter reactivity for the N-cadherin/catenin complex was due to a lesser amount of N-cadherin/catenin complexes expressed in WERI-Rb-1 cells and not to a failure to immunoprecipitate the N-cadherin/catenin complex in WERI-Rb-1 cells. In Y79 cells, there was only a faint reactivity for α- and β-catenin and no reactivity for N-cadherin. A more sensitive method was therefore used for the detection of an N-cadherin/catenin complex in Y79 cells. Autoradiography of a 7.5% SDS-polyacrylamide gel of N-cadherin, α-catenin, and β-catenin immunoprecipitates from lysates of cells labeled with ^{35}S methionine showed bands at 135 kDa (N-cadherin), 102 kDa (α-catenin), and 94 kDa (β-catenin) in the three immunoprecipitates from Y79 and WERI-Rb-1 cells (Fig. 2B). Flow cytometry of SK-N-SH, WERI-Rb-1, and Y79 cells confirmed these findings (Fig. 2C). N-cadherin was expressed by the three cell lines, with SK-N-SH showing the highest and Y79 the lowest intensity. WERI-Rb-1 and Y79 cells showed an irregular staining pattern with a disrupted honeycomb and clustered N-cadherin, α-catenin, and β-catenin, as seen in retinoblastoma tissue (Fig. 3).

The capping assay was performed to evaluate the strength of the association of the N-cadherin/catenin complex with the cytoskeleton.[45] At 4°C, fluorescent immunosignals indicating N-cadherin were equally distributed over the cell surface in the three cell types (Fig. 4). After a shift of temperature from 4°C to 37°C, the fluorescent signal gradually capped at one pole of WERI-Rb-1 and Y79 cells within 30 minutes of incubation,

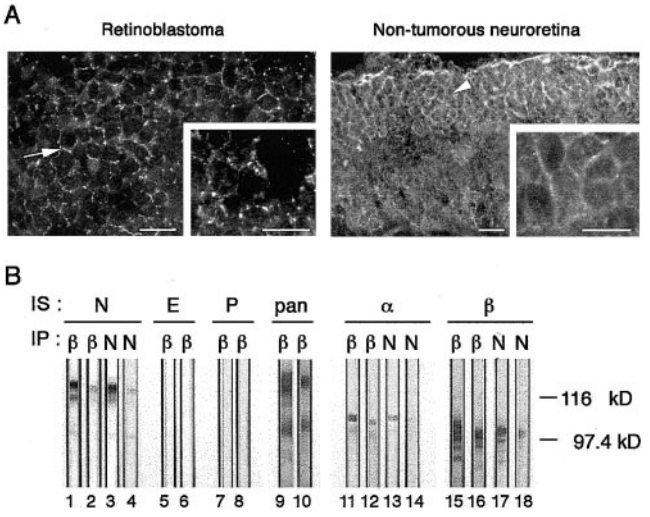

Figure 1. Normal retina and retinoblastoma express an N-cadherin–catenin complex. (**A**) Immunofluorescence micrographs of retinoblastoma and neuroretina stained for α-catenin. Frozen sections were immunostained with an antibody against α-catenin. Normal retina showed a regular honeycomb pattern (*arrowhead*). In retinoblastoma the honeycomb pattern was interrupted and showed clusters (*arrow*). (**B**) Western blot analysis of β-catenin (β) and N-cadherin (N) immunoprecipitates of lysates from the same neuroretina and retinoblastoma as shown in (**A**). Precipitated proteins were separated by 7.5% SDS-PAGE, transferred onto membrane, and immunostained (IS) with antibodies recognizing N-cadherin (N; 13A9), E-cadherin (E; HECD-1), P-cadherin (P), pancadherin (pan; CH-19), α-catenin (α), or β-catenin (β). *Even* and *uneven numbered* lanes represent retinoblastoma and normal retina, respectively. Scale bar, 20 μm.

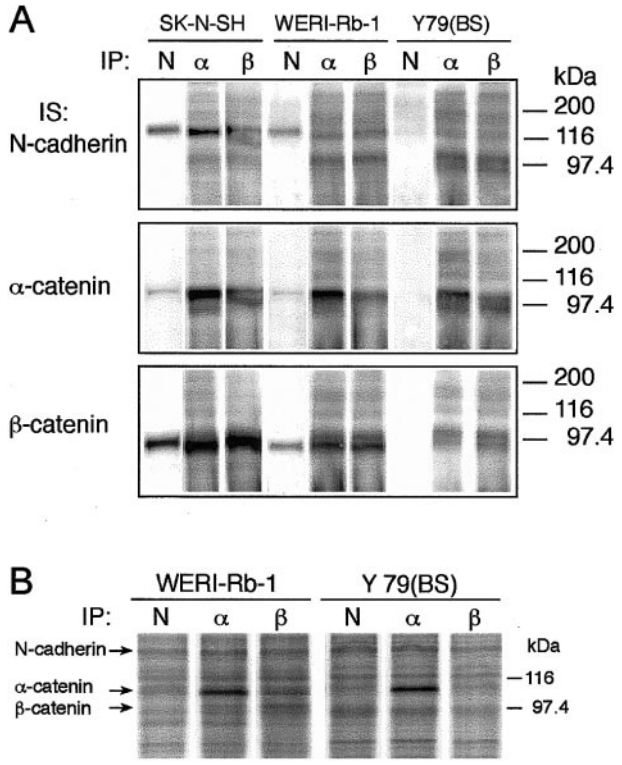

FIGURE 2. WERI-Rb-1 and Y79 retinoblastoma cells expressed an N-cadherin/catenin complex. (**A**) Western blot analysis of N-cadherin (N; CH-19), α-catenin (α), and β-catenin (β) immunoprecipitates (IP) from retinoblastoma cell lysates. SK-N-SH cells were used as a positive control for N-cadherin, α-catenin, and β-catenin expression. Precipitated proteins were separated by 7.5% SDS-PAGE, transferred onto membrane, and immunostained (IS) with antibodies recognizing N-cadherin (13A9), α-catenin, or β-catenin. (**B**) Autoradiographs of 7.5% SDS-polyacrylamide gels of lysates from retinoblastoma cells labeled with Tran^{35}S methionine, containing 70% L-methionine, [^{35}S], and immunoprecipitated (IP) with an antibody against N-cadherin (N; 13A9), α-catenin (α), or β-catenin (β). *Arrows*: positions of N-cadherin, α- and β-catenin in accordance with molecular weight. (**C**) Flow cytometry of SK-N-SH (S), WERI-Rb-1 (W), and Y79 (Y) cells, labeled with GC-4 antibody, which recognizes N-cadherin (dilution 1:30), followed by fluorescein-linked secondary antibody. Cells labeled with fluorescein-linked secondary antibody alone were used as blank (B) control. *Abscissa*: relative fluorescence intensity; *ordinate*: number of cells.

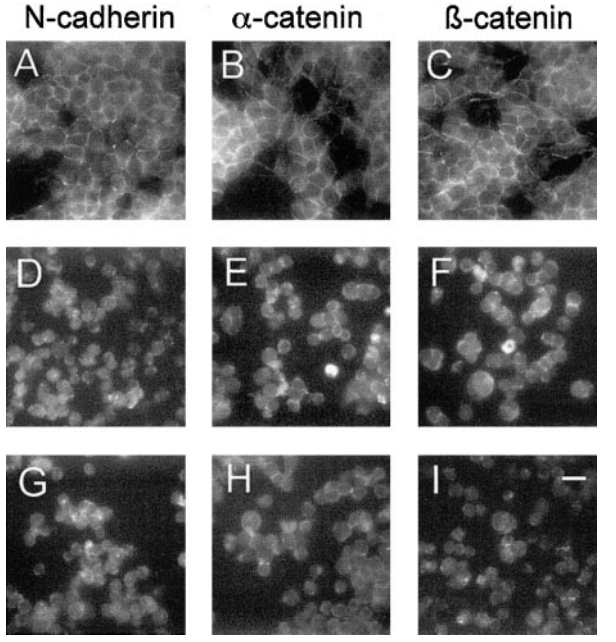

FIGURE 3. Neuroblastoma and retinoblastoma cells showed a different staining pattern for N-cadherin, α-catenin, and β-catenin. Immunofluorescence micrographs of SK-N-SH cells (**A**, **B**, **C**) showing a regular honeycomb pattern, and WERI-Rb-1 (**D**, **E**, **F**), and Y79 (**G**, **H**, **I**) cells showing an interrupted honeycomb pattern with clusters. Scale bar, 25 μm.

whereas it remained equally distributed over the surface of SK-N-SH cells. We concluded from this observation that in the retinoblastoma cell lines, the N-cadherin/catenin complex was not or was only weakly linked to the actin cytoskeleton.

Role of N-Cadherin in Aggregation and Invasion of Y79 and WERI-Rb-1 Cells

SK-N-SH, WERI-Rb-1, and Y79 cells aggregated after 30 minutes, with quantitative differences between cell types (Fig. 5). Treatment with GC-4 antibody inhibited aggregation of SK-N-SH and WERI-Rb-1, but not Y79 cells. WERI-Rb-1 and Y79 retinoblastoma cells do not attach to solid tissue culture substrate, but grow as loose aggregates in suspension. By contrast,

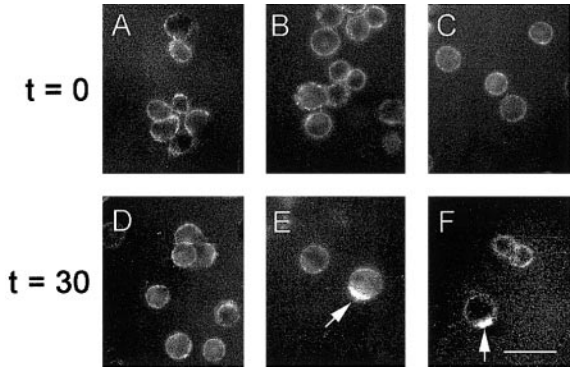

FIGURE 4. GC-4 antibody–mediated capping by N-cadherin in WERI-Rb-1 and Y79 cells. Immunofluorescence micrographs of living SK-N-SH (**A**, **D**), WERI-Rb-1 (**B**, **E**), and Y79 (**C**, **F**) cells. Cells in suspension were labeled at 4°C with GC-4 antibody recognizing N-cadherin, followed by rabbit anti-mouse FITC. Photographs were taken immediately after cells were brought to 37°C (t = 0) or 30 minutes later (t = 30). *Arrows*: capping. Scale bar, 20 μm.

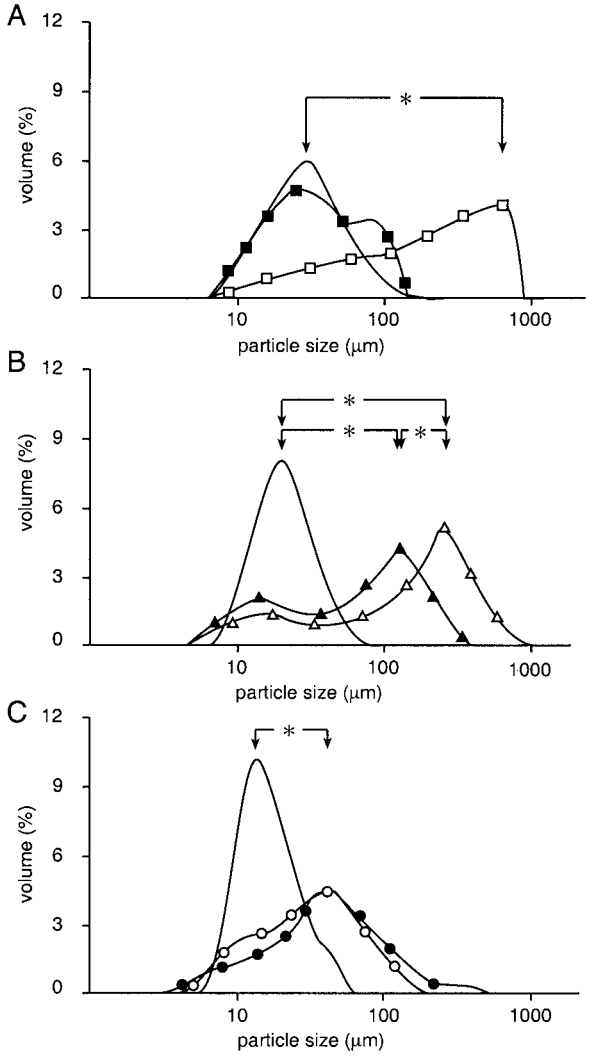

FIGURE 5. GC-4 antibody against N-cadherin inhibited aggregation of SK-N-SH and WERI-Rb-1, but not Y79 cells. Volume percentage distribution of the diameter of SK-N-SH (**A**), WERI-Rb-1 (**B**), and Y79 (**C**) aggregates. Cells were detached under cadherin-saving conditions, suspended in aggregation buffer, and allowed to aggregate for 30 minutes in the presence (*filled symbols*) or absence (*open symbols*) of GC-4 antibody. Cell aggregation was measured by particle size counting either after 0 minutes (*unsymboled traces*) or 30 minutes of aggregation. Komogorov-Smirnov statistics were used to analyze the differences between the cumulative particle size distribution curves. *Statistical difference for $P < 0.001$.

these cells attach to collagen type I, presumably through $\beta 1$ integrins,[46,47] and they invaded the collagen gel within 24 hours (Fig. 6). The GC-4 antibody, which functionally neutralizes N-cadherin,[48] significantly reduced the invasion index of ARM, SK-N-SH, Y79, and WERI-Rb-1 cells expressing chicken or human N-cadherin (Fig. 6A). The IgG1 isotype control antibody had no significant effect on invasion. The amount of GC-4 antibody needed to inhibit invasion was much lower for Y79 cells than for WERI-Rb-1 and SK-N-SH cells (Fig. 6B).

DISCUSSION

The N-cadherin/catenin complex, observed in normal retina, was disturbed in retinoblastoma, as evidenced by the irregular immunocytochemical staining pattern and the reduced levels of its elements in Western blot analysis. Such disturbed patterns have been described for the E-cadherin/catenin complex in most epithelial cancers, in which the complex serves as an invasion suppressor.[49] For tumors originating from N-cadherin–positive cells, the role of the N-cadherin/catenin complex in invasion is less well defined. In ovarian carcinomas, N-cadherin expression was used to define the origin of the tumor.[50] In astrocytomas and glioblastomas of the same histopathologic grade, no correlation has been found between

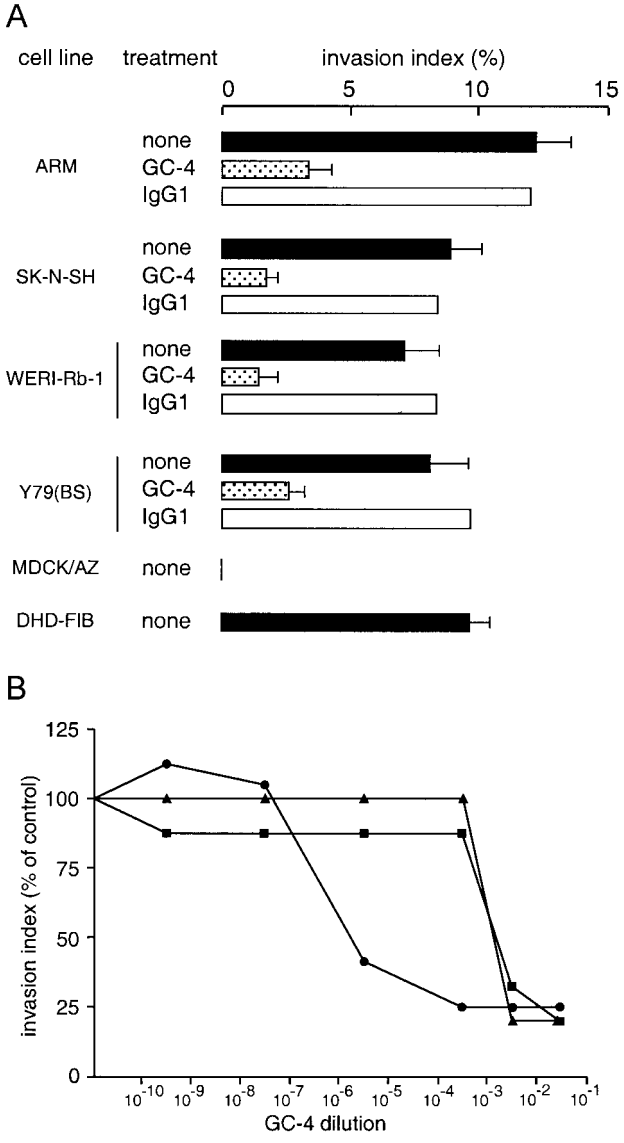

FIGURE 6. Collagen type I invasion was inhibited by the GC-4 antibody, which neutralizes N-cadherin. (**A**) Cells (1×10^5) were seeded on a layer of collagen type I without (*filled bars*) or with GC-4 antibody (dilution 1:30; *dotted bars*) or with IgG1 isotype control antibody (*open bars*). Invasion indices were calculated as the number of cells inside the gel relative to the total number of cells. Each bar represents mean data from three experiments, and flags show SDs, except for IgG1 antibody treatment, which represents one experiment. The invasion index of IgG1-treated cells differed from that of GC-4-treated cells but not from that of untreated cells (Student's *t*-test, $P < 0.005$). ARM and SK-N-SH cells served as a positive control for cells that expressed N-cadherin and invaded collagen type I. DHD-FIB and MDCK/AZ cells served as the control for cells that were invasive and noninvasive, respectively, of collagen type I. (**B**) Dose–response curve of inhibition of invasion of SK-N-SH cells (■), WERI-Rb-1 cells (▲), and Y79 cells (●) by GC-4 antibody. *Abscissa*: GC-4 antibody dilution; *ordinate:* invasion index expressed as a percentage of untreated cells.

invasion and amount of N-cadherin.[51] In neuroblastomas such a correlation has not been examined.[35] Transfection with cDNA-encoding N-cadherin rendered breast carcinoma cells invasive in synthetic basement membrane (Matrigel; BD Biosciences).[52] Our present observations show that N-cadherin promoted invasion of retinoblastoma cells, at least in vitro. Y79 and WERI-Rb-1 retinoblastoma cell lines expressed N-cadherin and invaded collagen type I. Invasion was inhibited by the GC-4 antibody that binds to the extracellular part of N-cadherin and functionally neutralizes the molecule.[39] Although Y79 cells expressed less N-cadherin than WERI-Rb-1 cells, their invasion index was the same. The amount of antibody needed to inhibit invasion, however, was proportional to the amount of N-cadherin expressed by the cells. Also in astrocytoma and glioblastoma cell lines, low expression of N-cadherin was compatible with high cellular motility.[51]

N-cadherin may support stable cell–cell adhesion and also stimulate migratory processes such as neurite outgrowth in vitro[53] and invasion in retinoblastoma. These apparently antithetic functions are explained by the fact that N-cadherin may be either mobile in the plane of the plasma membrane or may participate in the formation of stable adherens junctions that are linked to the cytoskeleton.[54] Indeed, in migrating neural crest cells, the bulk of N-cadherin molecules was found in the 2.5% Triton-soluble fraction. After treatment with tyrosine kinase or phosphatase inhibitors, stable N-cadherin–mediated intercellular contacts were restored.[25,55] Our experiments indicate that, in retinoblastoma, the majority of N-cadherin molecules are not firmly linked to the cytoskeleton. First, the GC-4 antibody caused capping of N-cadherin at one pole of Y79 and WERI-Rb-1 cells, as observed also for E-cadherin in human colon cancer cells HCT-8/E11R1 without α-catenin, an element that is necessary to link E-cadherin to the cytoskeleton (Marc Bracke, personal communication, May 2001).[56] Two, the disturbed immunohistochemical pattern of N-cadherin found by us in retinoblastoma resembled that in high-grade, highly invasive prostate cancer.[20]

Taken together, these observations suggest that in the normal retina, N-cadherin is coupled to the cytoskeleton and serves cell–cell adhesion as an element of adherens junctions, whereas in retinoblastoma N-cadherin is released from the cytoskeleton and participates in cellular invasion. Western blot analysis of detergent extracts with 0.1% and 1% Triton X-100 from colon cancer cells HCT-8/E11 and their α-catenin–negative variant HCT-8E11R1, human retinal pigment epithelial (RPE) cells at passage 5, retinoblastoma cells WERI-Rb-1, or Y79 did not contribute to our understanding. We found no or very minor differences in detergent solubility of N-cadherin, E-cadherin, β-catenin, α-catenin, or actin (Van Aken et al., unpublished results, 2001).

Several mechanisms may underlie stimulation of invasion by N-cadherin. In chick embryos, N-cadherin mediates outgrowth of retinal ganglion cells through homophilic, homotypic interaction with other neurons or homophilic, heterotypic interactions with other N-cadherin–expressing cells, such as oligodendrocytes.[57] Axon and dendrite outgrowth are impaired by transfection of the eye primordia with a mutant with a large deletion in the extracellular domain, but not with a mutant consisting of the cytoplasmic domain and competing for catenin binding, suggesting that the extracellular part, but not the catenin-binding part, of N-cadherin is essential for retinal outgrowth. The migration of single cells through synthetic basement membrane (Matrigel; BD Biosciences)–coated filters depended on the heterophilic interaction between N-cadherin and FGFR1[52] or the indirect interaction between N-cadherin and β1 integrin through phosphorylation cascades. N-cadherin–FGFR1 interaction has been extensively analyzed in neurite outgrowth of retinal ganglion cells and in pheochromocytoma cells.[27,53,58,59] *Cis* interaction between the HAV-binding motif of the fourth extracellular domain of N-cadherin and the HAV motif between the first and second immunoglobulin-like domain of FGFR1 results in increased mitogen-activated protein kinase (MAPK) activation and matrix metalloproteinase (MMP)-9 transcription.[60] The link with FGFR1 may be realized through transmembrane native N-cadherin or by N-cadherin fragments shed into the collagen.[61] N-cadherin fragments (90 kDa) are products of the naturally occurring proteolytic turnover that stimulate neurite outgrowth as potently as native N-cadherin molecules.[62] N-cadherin–β1 integrin interaction, as retinoblastoma cells bound to collagen type I through β1 integrins,[46] has been described in migrating retinal neurons and myoblasts.[28,55,63,64] It has been hypothesized that, in the developing chick retina, activation of a GalNAcPTase by its ligand neurocan results in translocation of Fer kinase from the N-cadherin/catenin complex to the β1 integrin complex. As in the N-cadherin/catenin complex, loss of phosphorylated active protein tyrosine phosphatase 1B would result in hyperphosphorylated β-catenin and the uncoupling of N-cadherin from the actin cytoskeleton. The β1 integrin complex would be inactivated by phosphorylation of p130cas-binding partners. As a result, the integrin complex is rendered nonfunctional and migration of neurites is inhibited.

In another in vitro assay using embryonic chick heart as a substrate, WERI-Rb-1 and Y79 cells failed to invade (Van Aken et al., unpublished results, 2001), in line with the findings of others.[65] This observation illustrates again the dependence of invasion on the elements of the host that may either stimulate or inhibit invasion.[66,67] Moreover, it cautions against extrapolation of our experimental observations to the complex situation in retinoblastoma in vivo.

The present aggregation experiments with WERI-Rb-1 and Y79 cells confirmed others' results.[30] In the latter and in our experiments, N-cadherin participated in the aggregation of WERI-Rb-1, but not Y79 cells, as evidenced by trituration in Ca^{2+}-free compared with Ca^{2+}-containing medium[30] and by aggregation in the presence or absence of neutralizing GC-4 antibody (our present experiments). Absence of inhibition by antibody in Y79 and partial inhibition in WERI-Rb-1 cells is evidence for the participation of other adhesion molecules, such as nerve cell adhesion molecule (NCAM) and L1, that belong to the Ca^{+2}-independent immunoglobulin superfamily. In conclusion, normal retina and retinoblastoma both express an N-cadherin/catenin complex. In normal retina, N-cadherin is localized at the adherens junctions serving cell–cell adhesion, whereas in retinoblastoma, N-cadherin is released from the cytoskeleton and participates in cellular invasion.

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